Using chemical probes to investigate the sub-inhibitory effects of azithromycin†

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The antibacterial drug azithromycin has clinically beneficial effects at sub-inhibitory concentrations for the treatment of conditions characterized by chronic Pseudomonas aeruginosa infection, such as cystic fibrosis. These effects are, in part, the result of inhibition of bacterial biofilm formation. Herein, the efficient synthesis of azithromycin in 4 steps from erythromycin and validation of the drug’s ability to inhibit biofilm formation at sub-MIC (minimum inhibitory concentration) values are reported. Furthermore, the synthesis of immobilized and biotin-tagged azithromycin analogues is described. These chemical probes were used in pull-down assays in an effort to identify azithromycin’s binding partners in vivo. Results from these assays revealed, as expected, mainly ribosomal-related protein binding partners, suggesting that this is the primary target of the drug. This was further confirmed by studies using a P. aeruginosa strain containing plasmid-encoded ermC, which expresses a protein that modifies 23S rRNA and so blocks macrolide entry to the ribosome. In this strain, no biofilm inhibition was observed. This work supports the hypothesis that the sub-inhibitory effects of azithromycin are mediated through the ribosome. Moreover, the synthesis of these chemical probes, and proof of their utility, is of value in global target identification in P. aeruginosa and other species.

Introduction

Antibiotics have had an essential role in the global increase in quality of life and life expectancy. The macrolide† class of antibiotics was discovered in the 1950s, and, as a result of their clinical efficacy and safety, now represents 20% of all prescribed antibiotics.‡ Macrolides show good in vitro activity against Gram positive bacteria and have been shown to inhibit protein synthesis by interacting with bacterial ribosomes.§ Azithromycin (1) (Fig. 1),¶ approved for clinical use in 1992 and marketed as Zithromax® by Pfizer, is one of the most commonly prescribed macrolides and is used to treat a range of bacterial infections including pneumonia and acute bronchitis.© However, azithromycin does not have a bacteriostatic or bactericidal effect in vivo against Gram negative bacteria, such as Pseudomonas aeruginosa (minimum inhibitory concentration [MIC] value >1000 μg ml⁻¹).® Nevertheless, over the last decade, azithromycin at sub-inhibitory concentrations (i.e. sub-MIC) has been shown to have clinically beneficial effects in cystic fibrosis (CF) sufferers.† In CF,‡ and other related pulmonary tract infections,§ chronic P. aeruginosa infection is the major cause of morbidity and mortality.© Although azithromycin is known to inhibit protein synthesis by blocking the protein exit tunnel in bacterial ribosomes,¶ its effects at lower concentrations, where it is neither bacteriostatic or bactericidal, are less well understood. Herein, we report the synthesis of azithromycin (1), the

Fig 1 The structures of azithromycin (1), the biotin-tagged analogue 2, and the immobilized equivalent 3.
Results and discussion

Azithromycin causes therapeutic effects at sub-inhibitory concentrations that are clinically relevant. The unresolved question is whether these sub-inhibitory effects are due to azithromycin targeting the ribosome or (as yet) unidentified proteins. We sought to test the hypothesis that azithromycin targets a non-ribosome related protein using the tagged analogues 2 and 3 in pull-down assays.

By adapting known methodologies we were able to synthesize azithromycin from erythromycin (4) in an overall yield of 32% over 4 steps (Scheme 1). Initially, erythromycin (4) was converted to the (E)-oxime 5 in 79% yield by a reaction with hydroxylamine in pyridine. Compared to alternative methods investigated, this reaction furnished exclusively the desired E-isomer and did not lead to formation of the α,β-unsaturated by-product 6. Using TsCl and NaHCO3 then facilitated the formation of the cyclic 6,9-iminoester 7 via an intercepted Beckman rearrangement of 5. Again, under these conditions, selective formation of 7 was observed in the absence of the alternative 9,11-iminoester product 8. The subsequent reduction of 7 to the secondary amine 9 could be achieved by H-Cube® hydrogenation or more productively with NaBH4. The synthesis of azithromycin was then completed under Eschweiler–Clarke methylation conditions.

In order to confirm that the biofilm inhibition assays were conducted at sub-MIC values, cultures of P. aeruginosa PAO1 (wild-type) were grown in the presence of azithromycin (3.9 μg ml⁻¹ to 1000 μg ml⁻¹). Under these conditions, using alanine-glycerol-salts (AGS) media, no growth inhibition was observed (i.e. MIC₅₀ > 1000 μg ml⁻¹). Full experimental details can be found in the ESI.† Biofilm inhibition assays with azithromycin were carried out in the same growth medium (Fig. 3a). These assays involved incubating...
Fig. 3  (a) Biofilm assays at various concentrations of azithromycin. After staining with crystal violet and sequential washing, any biofilm present retains the purple stain. The more purple the appearance of the well, the greater the biofilm formation. (b) Graph showing the absorption at 595 nm ($A_{595}$) against azithromycin concentration. The lower the absorption, the less biofilm is present. Both these figures show that azithromycin, at the concentrations investigated, inhibited biofilm formation.

the $P. aeruginosa$ PAO1 cultures with azithromycin (2.5 µg ml$^{-1}$ to 20 µg ml$^{-1}$) for 5 days at 37 °C in microtitre plates. The biofilms that resulted could then be visualized by crystal violet staining (purple) and the (relative) amounts quantified by analyzing the absorption from each sample at 595 nm ($A_{595}$). Azithromycin was shown to have a marked effect on the biofilms of $P. aeruginosa$ and inhibition was observed at all of the concentrations investigated (Fig. 3b, biofilm inhibition results in the wells appearing less purple).

In an effort to identify the molecular targets of azithromycin, the analogues 2 and 3 were synthesized for use in pull-down (affinity-chromatography) assays. The use of affinity methods is a common approach to target identification. The synthetic route to both 2 and 3 initially involved the conjugate addition of the azithromycin precursor 9 with acrylonitrile to give 10 followed by Raney nickel reduction (using an H-Cube$^5$) to give the tethered amine compound 11 (Scheme 2). Although a small amount of the dimer 12 (2%) was formed, these reactions proceeded in acceptable yields and provided a suitable site for attaching either biotin or a solid support. The biotinylated compound 2 was formed directly via an EDC–DMAP coupling of 11 with D-biotin (Scheme 2). To attach 11 to the solid support, two further steps were required. The amine 11 was initially converted to the $\alpha$-azoamide 13 in an EDC–DMAP coupling with the acid 14. The solid support 15 was derivatized with an alkyne functionality. The azide compound 13 could therefore be immobilized using ‘click chemistry’. The highly efficient copper-catalyzed Huisgen 1,3-dipolar cycloaddition thus furnished the immobilized azithromycin compound 3 (Scheme 2).

In an effort to test the hypothesis that azithromycin acts to inhibit biofilm formation in $P. aeruginosa$ by acting on a protein target other than the ribosome (maybe one involved in quorum sensing), pull-down investigations were performed using suitably designed negative controls to account for non-specific binding interactions. The biotin-tagged compound 2, immobilized via binding to solid supported streptavidin, gave inconclusive results due to the tag binding to various biotin-binding proteins in the cell. However, the immobilized azithromycin analogue 3 gave more promising results and the resulting 1D PAGE gel showed clear enrichment of specific bands (Fig. 4) compared with samples using control sepharose beads such as 15. A small number of

Scheme 2  The synthesis of the biotin-tagged and immobilized azithromycin compounds 2 and 3.
strongly-stained bands, presumably corresponding to highly-abundant captured proteins, were excised from the gels and digested with trypsin prior to MS/MS analysis to determine the identity of the protein(s). Most of the proteins identified this way were directly associated with ribosome function, translation or the folding of nascent proteins. However, we also found that the immobilized azithromycin captured RNA polymerase β subunit, although this might not be unexpected in a tightly coupled prolaryotic transcription–translation system such as that found in many rapidly growing species like *P. aeruginosa*.

Since there was no evidence that analogue 3 was binding any proteins other than those associated with translation, this suggested that indeed *only* the ribosome was critical to azithromycin’s ability to inhibit biofilm formation at sub-MIC values. To further test this hypothesis we performed biofilm inhibition assays in a strain of *P. aeruginosa* that contained the plasmid-encoded ermC. Plasmid-encoded ermC (permC) was constructed by sub-cloning the ermC gene from permCT into pUCPKS to enable stable maintenance in *P. aeruginosa*. The ermC gene encodes a methylase which acts to modify the ribosome at a known macrolide binding site (specifically a single adenine residue (A2058 in *Escherichia coli*) in the 23S rRNA). This modification makes the bacteria less susceptible to the action of macrolides and is one of the modes of action by which bacteria become resistant to this type of antibiotic (the other is via increased drug-efflux). Performing the biofilm assays under the same conditions as before (Fig. 2) but using the permC-containing *P. aeruginosa* strain gave notable results: little or no biofilm inhibition was observed (Fig. 5). This result clearly indicated that an azithromycin–ribosome interaction is required for biofilm formation to be affected at sub-MIC values. Similar results have been observed previously using the *P. aeruginosa* PA01 strain expressing the 23S methylase ErmBP.

Conclusions

An efficient synthesis of azithromycin was developed, and this material was used to validate the drug’s ability to inhibit biofilm formation at sub-MIC values for *P. aeruginosa*. Biotin-tagged and immobilized azithromycin derivatives were synthesized and exploited in pull-down assays in an effort to identify binding partners and hence illuminate its sub-inhibitory mode of action.